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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 33/543, A61K 47/48 — A1

(11) International Publication Number: **WO 99/17120**

(43) International Publication Date: 8 April 1999 (08.04.99)

(21) International Application Number: PCT/US98/19716

(22) International Filing Date: 21 September 1998 (21.09.98)

(30) Priority Data:
08/938,986 26 September 1997 (26.09.97) US

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(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PREPARING CONJUGATES USING POLYETHYLENE GLYCOL LINKERS

(57) Abstract

The instant invention presents a rapid simple method for preparing solid phases, preferably beads, with antigens or other substituents presented on the surface in such a manner that the antigens/substituents retain their original functionality and conformation, as well as much of their native structure, to permit their use in a wide array of applications. Specifically, the substituent is attached to the surface of the solid phase by using a bifunctional derivative of polyethylene glycol. The polyethylene glycol (PEG), it has been found, acts not only to facilitate the attachment of the substituent to the solid surface, but also acts as a buffer to prevent or reduce any interaction of the solid surface with the attached substituent or, indeed, with any other biological compounds to which it may become exposed during the use of the solid surface conjugates.

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PREPARING CONJUGATES USING POLYETHYLENE GLYCOL LINKERS

BACKGROUND OF INVENTION

Specific and high affinity molecular recognition is critical for a variety of diagnostic applications. The predominant recognition methodology involves the use of molecular species which recognize the target. For example, protein molecules that recognize a specific target can be generated as antibodies. Such antibodies can then be used to recognize the target and bind to it. By use of appropriate labeling techniques, the bound target can be identified and/or separated from the remaining population. Among such separation techniques, flow cytometry is uniquely suited for such applications due to its capability to perform simultaneous multiparameter analysis and to separate (or sort) unique cell populations from heterogeneous mixtures of cells.

Other techniques similarly utilize this technology. However, in each, the critical parameter is the recognition of the antigen or target. This is strongly affected by the conformation of the target, and, in general, it is essential to have the target in a confirmation resembling its native conformation.

As conformation is strongly influenced by the environment of a molecule, it becomes extremely difficult to attach a target molecule (or substituent) to a bead or other structure to facilitate its use in a desired application, yet retain a confirmation similar to its native conformation.

In addition, for many applications in flow cytometry, it is useful to utilize beads having other substituents such as fluorophores attached to the surface. Thus, it would be useful to have an attachment methodology which is also amenable to attaching such other substituents to the surface of the beads in addition to the antibodies.

SUMMARY OF INVENTION

The instant invention presents a rapid simple method for preparing solid phases, preferably beads, with antigens or other substituents presented on the surface in such a manner that the antigens/substituents retain their original functionality and conformation, as well as much of their native structure, to permit their use in a wide array of applications. Specifically, the substituent is attached to the surface of the solid phase by using a bifunctional derivative of polyethylene glycol. The polyethylene glycol (PEG),it has been found, acts not only to facilitate the attachment of the substituent to the solid surface, but also acts as a buffer to prevent or reduce any interaction of the solid surface with the attached substituent or, indeed, with any other biological compounds to which it may become exposed during the use of the solid surface conjugates.

Thus, the polyethylene glycol has a dual function, the first being that of a linking group facilitating the attachment of the substituent to the solid phase, and the second being that of maintaining, to the greatest extent possible, the native conformation of the substituent. While not wishing to be bound by theory, it is believed that this is the result of three properties conveyed by the PEG, namely: additional flexibility of the attached components due to the linking group; a reduction in the conformational distortion of the substituent due at least partially to a reduction in the "sticking" of the other portions of the substituent to the solid surface; and a reduction in the non-specific binding of other proteins and similar materials to the solid surface.

DETAILED DESCRIPTION OF THE INVENTION

The complexes of the instant invention are formed by attaching the substituent(s) to the solid phase by the use of the PEG as a bifunctional derivative to link the substituent to the solid phase. As such, the solid phase must have a surface functional group available to facilitate the bonding of the PEG. In a preferred embodiment of this

invention, the solid phase is a bead which possesses amine (NH2) functionalities. A preferred bead of this type is a 6 micron polymethylmethacrylate (PMMA-NH2) bead. However, the choice of the solid phase will be governed by the specific applications intended.

A variety of substituents can, thus , be attached to the solid phase by this methodology. Such substituents include, but are not limited to, fluorophores (such as phycoeryhtrin), streptavidin (which can be used to bind biotinylated ligands), oligonucleotides, and biological compounds such as proteins and nucleic acids. Indeed, so long as the substituent will bind, or can be chemically modified to permit it to bind, to the PEG, the methodology can be utilized.

The PEG utilized must be of a sufficient size to convey the desired properties, but not so large as to significantly hinder the kinetics of derivitization, attachment of the substituent, or the functionality of the attached substituents. In general, a molecular weight of 1,000-5,000, more preferably 2,000-3,000, and even more preferably 2,000 would be used, but it is o be understood that PEG polymers of even higher and/or lower molecular weights can be used depending on the particular application. While the preferred heterobifuctional reagent of this invention, succimidyl propionate-PEG-2000-orthopyridyl-disulfide (OPSS-PEG2000-SPA) is available commercially (Shearwater Polymers, Huntsville, AL), other functional moieties on the PEG termini may be used as the particular application dictates. The benefits of the OPPS-PEG2000-SPA are two-fold, namely, to activate the solid surface in order to facilitate the attachment of the substituent, and to provide a "barrier layer" between the solid phase and the substituent(s) on the surface or components in the surrounding medium.

Similarly, the functional groups on the PEG reagent will be selected based on the chemistry of the solid surface and the desired functionality for substituent attachment. In a preferred embodiment, the solid phase will have a primary amine group (preferably a PMMA-NH2) and the one end of the PEG molecule will have a succinimidyl group,

thereby facilitating formation of an amino ester (peptide) linkage between the solid surface and the PEG molecule. Further, in this preferred embodiment, the PEG molecule has a protected sulfhydryl group (preferably orthopyridyl-disulfide) which can be deprotected by reduction with DTT and, thus, be available to form a thioether link with a maleimide derivatized substituent or, which could react, intact, with a sulfhydryl derivatized substituent by disulfide interchange.

The use of the PEG attachment methodology to provide a PEG spacer between the solid phase and the substituent also will facilitate the binding of ligands or molecules to the substituent, help to retain the native properties of the bound substituents; and enhance mobility, and thus accessibility, of the attached substituents. Further, the specificity of bonding to the substituent(s) is enhanced, because the PEG linker provides a "barrier" to reduce the non-specific binding of other species to the solid surface.

Examples

The following examples illustrate certain preferred embodiments of the instant invention, but are not intended to be illustrative of all embodiments.

Example 1 - Preparation of phycoeryhtrin coated beads with a PEG 2000 linker

Polymethacrylate amino beads (6.1 microns PMMA-NH_2), at a concentration of 1.5% w/v, were admixed with OPSS-PEG2000-SPA, at a concentration of 5 mM, in an aqueous buffer containing 50 mM sodium phosphate/150 mM sodium chloride/1 mM EDTA/0.01% w/v Tween 20, adjusted to a pH of 7.7, and reacted at room temperature for 2 hours. The beads were subsequently washed with reaction buffer, reduced with 25mM dithiothreitol, and subsequently washed with 50 mM MES (2-(N-morpholino)ethanesulfonic acid) /150mM sodium chloride/1 mM EDTA/0.01 %w/v Tween 20 adjusted to a pH of 6.0.

The resultant beads were then reacted at room temperature fro 2 hours with PE (phycoerythrin)-maleimide and BSA-maleimide (both PE and BSA maleimide were synthesized using succinimidyl 4-(N-maleimido-methyl)-cyclohexane-1-carboxylate (SMCC) (Pierce Biochemical Corp) to arrive at a protein to bead ratio of 5 micrograms per square centimeter of bead surface area. The ratio of maleimide PE to maleimide BSA was adjusted to achieve the desired number of PE molecules per bead. Residual -SH groups were capped by reacting with 0.5 mM N-ethylmaleimide for 15 minutes at room temperature. The resultant beads were washed into PBS/0.1% sodium azide/0.2% gelatin/0.01% w/v Tween 20.

The beads prepared by this method exhibited more uniform fluorescence and unaltered emission characteristics (essentially indistinguishable from that of PE-conjugated antibodies)and greater stability than PE beads made with shorter linkers.

Example 2 -Preparation of Streptavidin Beads with a PEG2000 Spacer

The beads were prepared by the same synthetic procedure as described in Example 1, except that the OPSS-PEG2000-SPA labeled, reduced, and washed beads were reacted with streptavidin maleimide at 5 micrograms per square centimeter bead surface area.

The beads prepared by this method had about twice the ligand binding capacity (biotin-PerCP) as did conventional beads with a shorter spacer. While not wishing to be bound by theory, it is postulated that this is due to the greater accessibility of streptavidin on the PEG spacer, versus streptavidin held onto the bead surface with a shorter spacer. Further, non-specific binding (e.g. IgG1 to PerCP or PE) was very low; this was attained without the use of blocking buffers or proteins.

Example 3 -Synthesis of oligonucleotide beads with a PEG2000 spacer

The beads were prepared by the same synthetic procedure as described in Example 1, except that the OPSS-PEG2000-SPA labeled, reduced, and washed beads were reacted with 120 pmole/cm2 of 5'-maleimido oligonucleotide, which was previously synthesized by the reaction of the 5' NH2-22 mer oligonucleotide with SMCC.

The beads prepared by this method bound FITC labeled complimentary oligonucleotide with a low CV and low non-specific binding.

Example 4 - Preparation of Bead Conjugates using other PEG linkers

Bead conjugates were made using the synthetic procedure of Example 1, except that PEG1000 and PEG 20,000 were substituted for the PEG2000. It was found that the PEG1000 gave most of the benefits found with the PEG2000, except that the syntheses were not always reproducible. the PEG 20,000 gave acceptable results, but was difficult to synthesize due to the high viscosity of the reaction solution and slower reaction kinetics.

It is apparent that many modifications and variations of this invention as hereinabove set forth may be made without departing from the spirit and scope thereof. The specific embodiments are given by way of example only and the invention is limited only by the terms of the appended claims.

WHAT IS CLAIMED IS:

1. An improved method for conjugating a substituent to a solid surface comprising attaching a bifunctional derivative of a polyethylene glycol to said solid surface and said substituent such that said bifunctional derivative of polyethylene glycol is a linking agent between said solid surface and said substituent.

2. The method of Claim 1 wherein the solid surface is a bead.

3. The method of Claim 2 wherein the bead possesses amine surface functionalities.

4. The method of Claim 3 wherein the bead is a polymethylmethacrylate bead.

5. The method of Claim 1 wherein the substituent is selected from the group consisting of fluorophores, streptavidin, and biological compounds.

6. The method of Claim 5 wherein the substituent is a fluorophore selected from the group consisting of phycoerythrin and perdinin chlorophyll protein.

7. The method of Claim 5 wherein the substituent is a biological compound selected from the group consisting of proteins and nucleic acids.

8. The method of Claim 7 wherein the substituent is an oligonucleotide.

9. The method of Claim 7 wherein the substituent is an antibody.

10. The method of Claim 9 wherein the antibody is a monoclonal antibody.

11. The method of Claim 1 wherein the polyethylene glycol has a ? molecular weight of about 1,000-about 5,000.

12. The method of Claim 11 wherein the polyethylene glycol has a ? molecular weight of about 2000.

13. The method of Claim 1 wherein the polyethylene glycol is derivatized with a maleimide compound.

14. The method of Claim 13 wherein the maleimide compound is succinimidyl propionyl dithiopyridine.

INTERNATIONAL SEARCH REPORT

tn. ational Application No
PCT/US 98/19716

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/543 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12Q A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	EP 0 874 242 A (RANDOX LAB LTD) 28 October 1998 see claims 12-20 see page 3, line 41 - line 43 see page 5, line 47 - line 50; figure 5 ---	1-14
P,A	WO 98 32466 A (FRANCIS GILLIAN ELIZABETH ;FISHER DEREK (GB); MALIK FAROOQ (GB); P) 30 July 1998 see claims 1,2,6-12 see page 9, line 9 - line 16 ---	1-14
P,X	EP 0 806 250 A (BOEHRINGER MANNHEIM GMBH) 12 November 1997 see claims 6-10 see page 4, line 7 - line 23 --- -/--	1-14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
28 January 1999	08/02/1999

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

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